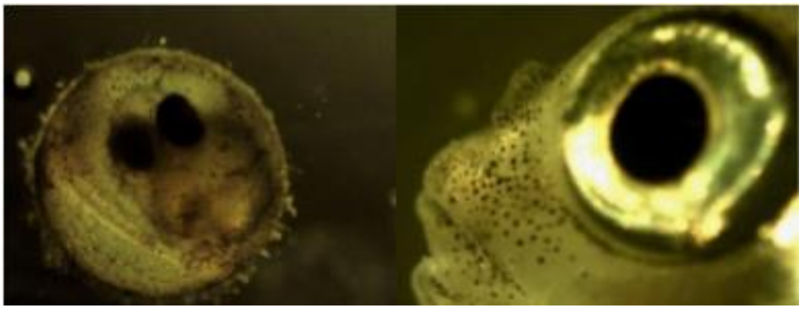

Internship at Aquário Vasco da Gama

Description of an updated *ex-situ* conservation protocol for endangered portuguese cyprinids

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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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MSC

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Abstract

Iberian Peninsula is a *hot spot* of biodiversity regarding freshwater fish species and contains the highest proportion of endemic species in Europe. This distinct fish assemblage is the result of past geological events that shaped the Iberian basins into a closed system of endorheic basins, that represent the main dispersion barrier to fish dispersal, which may have favoured allopatric speciation. Iberian ichthyofauna are highly vulnerable to habitat alterations due to their reduced distribution range and low genetic diversity. About 70% of Iberian freshwater species are characterized as threatened, according to IUCN criteria, due to severe habitat degradation, water pollution and introduction of exotic species, as freshwater ichthyofauna elsewhere. Conservation measurements for these species are urgent to guarantee their survival, habitat restoration and improve water quality.

Aquário Vasco da Gama (AVG), Quercus (NGO) and ISPA (University) developed a pioneer project regarding the *ex-situ* conservation of critically endangered species endemic to Portugal. To ensure the reproductive success, this protocol follows a 'naturalistic approach'. Through environmental enrichment strategies, this approach aims to avoid the domestication of the species and is based on: (1) outdoor tanks with natural photoperiod conditions; (2) naturally occurring spawning, fish were provided with spawning natural substrates; (3) availability of shelter and maternity areas, no human intervention for protection of larvae, fry and/or juveniles, to promote the natural behaviour of finding shelter and avoiding predators and (4) minimal human intervention to minimize fish stress.

The main goal of this internship at AVG was to describe the procedures of this *ex-situ* conservation project. From the broodstocks maintenance conditions to their release in the wild habitat, this work describes all the tasks performed at AVG for the conservation of four threatened populations of the endemic fish species: *Achondrostoma occidentale* (Western Ruivaco) (Sizandro and Sarafujo rivers), *Iberochondrostoma lusitanicum* (Sado river) and *Iberochondrostoma almacaii* (Arade river). Furthermore, this work provides the first descriptions on the early life stages of *A. occidentale*. By providing important key events and features, this data will enable the identification of this endangered species in future larval studies.

In summary, *ex-situ* conservation programs are a valuable tool for the survival of Iberian endangered species. However, should be applied as the last survival hypothesis and elimination of the main threats must be prioritized.

Key words: *Ex-situ* conservation; naturalistic approach; endangered fish; Iberian ichthyofauna

Resumo

A Península Ibérica é considerada um *hot spot* de biodiversidade no que diz respeito à ictiofauna de água doce e apresenta o maior grau de endemismo registado na Europa. Esta distinta biodiversidade resulta dos efeitos de eventos geológicos, que moldaram a estrutura hidrográfica da Península Ibérica num conjunto de bacias isoladas, que representam a principal barreira à dispersão, o que pode ter favorecido a especiação alopátrica. Devido ao isolamento geográfico e à baixa diversidade genética, estas espécies são extremamente vulneráveis a alterações nos ecossistemas. Cerca de 70% da ictiofauna Ibérica encontra-se com o estatuto de conservação '*Ameaçado*' na Lista Vermelha da IUCN, devido à severa degradação do habitat, poluição da água e introdução de espécies exóticas. Medidas de conservação para estas espécies são urgentes para garantir a sua sobrevivência, restauração do habitat e, consequentemente, melhorar a qualidade da água dos ecossistemas fluviais.

O Aquário Vasco da Gama (AVG) em conjunto com a Quercus (ONG) e o ISPA (Universidade) desenvolveram um projeto pioneiro de conservação *ex-situ* para espécies criticamente ameaçadas, endêmicas a Portugal. De modo a manter a integridade genética das populações e evitar o relaxamento dos processos de Seleção Natural, este protocolo segue uma "abordagem naturalista". Este método baseia-se em: (1) a manutenção das espécies em condições naturais de fotoperíodo e oscilações naturais de temperatura e pluviosidade, (2) promover a reprodução natural, através de substratos de posturas idênticos ao habitat natural, (3) disponibilidade de refúgios para larvas e juvenis, nenhuma intervenção humana para a proteção dos primeiros estágios de vida de modo a promover o comportamento natural de encontrar abrigo e evitar predadores e (4) mínima intervenção humana.

O principal objetivo deste estágio foi descrever os procedimentos deste protocolo de conservação. Desde a monitorização diária das condições de manutenção dos reprodutores até à sua libertação no habitat natural, neste relatório são descritas todas as etapas para a conservação de quatro populações ameaçadas nos sistemas fluviais de Portugal: *Achondrostoma occidentale* (rios Sizandro e Sarafujo), *Iberochondrostoma lusitanicum* (Rio Sado) e *Iberochondrostoma almacaii* (rio Arade).

Durante este estágio, foi possível, também, seguir e descrever as etapas do desenvolvimento embrionário e larvar para a espécie *A. occidentale* (Sizandro). Através de um método de monitorização ‘naturalista’, foram descritos os principais eventos e características relacionados com o desenvolvimento desta espécie, que fornecerá bases para futuros estudos.

Medidas de conservação *ex-situ* podem ser uma mais valia para a conservação destas espécies. Contudo devem ser aplicadas como a última hipótese de evitar a extinção efetiva das populações naturais. Esforços que visem a reabilitação das condições do habitat natural devem ser priorizados, de modo a contribuir para o bom estado ecológico dos ecossistemas fluviais.

Palavras-chave: Conservação *ex-situ*; abordagem naturalista; espécies ameaçadas; ictiofauna Ibérica.

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Glossary

AVG – Aquário Vasco da Gama

AR – Arade

DO – Dissolved Oxygen

FL - Fork Length

FVM – Faculty of Veterinary Medicine

IUCN – International Union for Conservation of Nature

ICNF - Institute for Conservation of Nature and Forestry

SA – Sado

SF - Safarujo

SZ – Sizandro

TL – Total Length

UN- United Nations

WFD - Water Framework Directive

WWF – World Wildlife Fund

1. Introduction

Freshwater ecosystems (i.e. lakes, ponds, rivers streams and wetlands) are highly rich in biodiversity and valuable for human well-being. They cover less than 1% of the global surface yet support over than 25% of all described vertebrates (WWF, 2018). They are crucial for sustainable development as they provide several services such as water for consumptive use, habitats for aquatic life, water purification and other regulatory and cultural services (Green *et al*., 2015).

These distinct ecosystems are under high levels of threat. According to the UN report 'Freshwater Strategy 2017-2021', freshwater ecosystems are one of the most threatened in the world. They are strongly affected by habitat fragmentation and destruction, invasive species, pollution, forestry practices and climate change.

In some regions, the interaction of this threats led to sharp decline in freshwater biodiversity, especially regarding fish species. In fact, in the 20th century freshwater ichthyofauna had the highest extinction rate worldwide among vertebrates (IUCN, 2016).

Iberian Peninsula is one of the most critically regions in Europe (Freyhof & Brooks, 2011). The unique assemblage of freshwater species that inhabited the river streams and the tradition of human activities in the Iberian waters, make the Iberian Peninsula a very interesting place for the study of pressures and conservation of freshwater fish species. However, knowledge of Iberian freshwater ichthyofauna is scarce and their conservation *status* is alarming (Rodeles *et al*., 2016). Because, most of these species have no economic or social value, there is just a few funding to support projects for their conservation and habitat protection (Doadrio, 2001). Hence, Iberian stream rivers have been subject to the cumulative effects of pollution and other anthropogenic pressures compromising water quality and the survival of these species.

The EU Water Framework Directive (WFD) refers to the idea that to ensure water quality for the uses in the future, freshwater ecosystems must be protected through their ecological improvement and restoration. The focus must be on the river basin scale by supporting the achievement of favourable conservation status of species. Therefore, conservation efforts are crucial to achieve the WFD goal. This report is the result of an internship at Aquário Vasco da Gama (AVG), that is one of the institutions that supports a pioneer conservation program regarding the conservation of Iberian freshwater fish species.

2. Iberian freshwater fish: Why are they so unique and so vulnerable?

2.1. Iberian Peninsula geomorphological history

Iberian Peninsula is considered a *hot spot* of biodiversity regarding freshwater fish species (Reyjol *et al*., 2007). The Iberian fish fauna is characterized by a high degree of diversification at the species level and the greatest level of endemism in Europe, comprising 73% of endemic species (composed mainly of small to medium sized Cyprinid species). (Cabral *et al*., 2005, Rogado *et al*., 2005). This unique richness is a result of a set of multi-scale events that promoted the distribution and abundance patterns of this species. In the absence of anthropogenic pressures, the dispersion, extinction and speciation of freshwater fish depends on changes in the hydrographical networks and consequent environmental fluctuation in their habitat. Hence, contemporary and historical events have played an important role in shaping the current genetic population structure of Iberian fish species (Filipe *et al*., 2009).

First records of cyprinids colonization in Iberian Peninsula dated to the end of the Oligocene (28 Ma). At this time Iberian's ichthyofauna was quite distinct from the current one, composed mainly by tropical fish fauna, Siluforms and Characiforms (De la Pena, 1995).

At the Miocene and Pliocene (11 -5 Ma) the hydrographical configuration of the Iberian Peninsula was formed. Along with active geoctonic faults, the basins were shaped into a closed system of "biogeographical Islands" (Calvo *et al*., 1993). These endorheic basins (i.e. drainage basin that retains water and allows no outflow to other external bodies of water) represent the main dispersion land barrier to fish dispersal, which may have favoured allopathic speciation of Iberian ichthyofauna (Robalo *et al*., 2006).

In addition, the continuous evaporation of Mediterranean Sea during the Missianian Salinity Crisis restricted the viability of marine habitats, which might have led to a forced adaptation of some species to a freshwater environment (Hewitt, 2000). Following the reflooding of the Mediterranean Basin trough the Gibraltar Strait, some species retained their freshwater habitats remaining isolated from the euryhaline Ponto-Caspian group of species.

At the Quaternary period, the Iberian Peninsula was influenced by successive cold periods events. Particularly, during the Last Glacial Maximum (16,000 – 18,000 years), the norther Europe was partial covered by ice, leading to a southward's movement of some mammals and bird species towards the Peri-Mediterranean (Hewitt, 2000). Since freshwater fish were unable to migrate along a north–south axis some species becoming extinct. In addition, a significant fluctuation of the sea level along the Atlantic cost was recorded – 140m below the contemporary level – leading to a possible formation of new dispersal paths for freshwater fish due to the downstream interconnection between adjacent rivers (Dias *et al*., 2000).

In summary, historical events such as the raise of the Iberian basins boundaries and glaciations phenomena contribute to the current complex Iberian fluvial network leading to the formation of a high number of independent river basins where the different species populations are strongly isolated and therefore highly vulnerable to habitat alterations (Clavero *et al*., 2004).

2.2. Conservation *status* of Iberian freshwater fish fauna

According to the last assessment by IUCN, about 70% of Iberian freshwater species are characterized as threatened. This region recorded highest proportion of species in the brick of extinction in Europe (*figure 1*) (Freyhof & Brooks, 2011).

Based on IUCN criteria, about 52 % of Iberian native fish species are considered as *critically endangered*, *endangered*, or *near threatened*. This conservation *status* is a result from the interaction of several anthropogenic pressures (Maceda-Veiga 2012). In addition, due to their limited geographic distribution range and low genetic diversity (Cabral *et al*., 2005, Sousa-Santos *et al*., 2014) these species have low chances of adaptability to habitat changes and fluctuations.

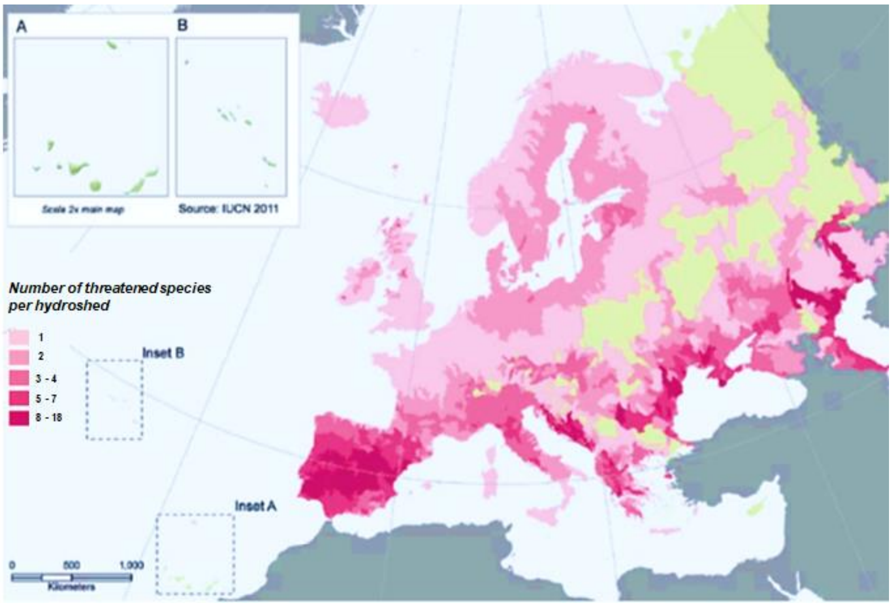

***Figure 1*- Distribution of threatened freshwater species in Europe per hydroshed.** Iberian Peninsula has one of the largest proportions of endangered species in Europe. (adapted from IUCN assessment, Freyhof & Brooks, 2011).

2.2.1. Main threats

i. Hydrological infrastructures and water extraction

Hydrological infrastructures associated with water extraction are the main cause for habitat disruption of Iberian freshwater fish (Johnson *et* al., 2008). In fact, an increased number of hydrological infrastructures were built in the last century to demand agricultural, hydropower and domestic needs (Maceda-Veiga,2012). These structures can cause hydrological changes on the flow regime and river function (e.g. water flow and temperature fluctuations) and therefore directly impact the survival chance of fish species. In addition, can promote the destruction of spawning grounds and enhance the introduction of exotic species.

ii. Introduction of exotic fish species

Iberian Peninsula is considered a *hot spot* region of exotic fish species. In fact, about 42 % of Iberian freshwater fish are non-native fish species (Maceda-Veiga, 2012) . The introduction of non-native fish species is also associated with increased risk of extinction as they can directly impact the survival rate of native species through: (1) competition for particular habitats or food sources (Maceda-Veiga, 2012), (2) hybridization phenomena (Doadrio, 2001), (3) transference of pathogens and diseases (Leunda, 2010), (3) alterations in ecosystem dynamics, such as impact on trophic connections and nutrient cycle and (4) changes in behavioural interactions and predation (Gozlan *et al*., 2010).

iii. Riparian vegetation disturbance

Riparian zones of Iberian Peninsula have been widely stripped due to the need for agricultural, and industrial zones (Sabater *et al*., 2009). Riparian vegetation is crucial for the ecological dynamics of the rivers and streams (Maceda-Veiga, 2012) and a bad management and disturbance can negatively impact Iberian ichthyofauna. This vegetation can contribute by: (1) providing habitat and nutrients diversification (e.g. roots and fallen branches can act as shelter and maternity grounds for fish), (2) acting as filter by removing pollutants and (3) controlling water temperature and evaporation rates by providing shadow areas (Flores *et al*., 2011).

iv. Water pollution

Iberian rivers have been negatively impacted due to water pollution resulting from the sewage effluent from agricultural, domestic and urban activities. Sabater *et al.,* (2009) described that more than 19 % of groundwater and over 50 % water stored in Iberian reservoirs were affected by an input of excess nutrients. This uncontrolled discharges into waterways can lead to eutrophication events promoting oxygen depletion and therefore compromising species survival (Damásio *et al*., 2007).

In summary, Iberian ichthyofauna faces a variety of anthropogenic pressures that represent the main reason for endemic fish sharp decline. Due to the interaction between the threats and the influence of climate change this species faces a low chance of survival (Maceda-Veiga, 2012) . In addition, most of these species have no economic or fishing value (Doadrio, 2001). Therefore just a few funds are available for the conservation of these species. Development of conservation and protection measures for these species and their wild habitat is urgent in order to prevent the extinction of Iberian ichthyofauna.

3. *Ex-situ* conservation programs: a tool for restocking endangered fish species

Supportive breeding programs have become a viable strategy for the protection and conservation of vulnerable and endangered species facing reduced natural populations or/and extremely deteriorated habitats (Faria *et al*., 2010). In this cases, *ex-situ* reproduction measures (i.e. the conservation of all levels of biological diversity outside the natural habitats) can be the only solution to prevent their extinction until the restoration of the conditions of the wild habitat (Maitland & Morgan, 2002). This reproduction strategy has as its main objective the release of captive bred in the streams where their founders were collected.

Ex-situ conservation programs have been applied in several species with low effective natural population sizes (Adamski *et al*., 2007; Bice *et al.,* 2013), particularly for the highly endangered European sturgeon species such as: *Acipenser oxyrinchus* (Kolman *et al.,* 2011), *A. naccarii* (Boscari & Congiu, 2014) and *A. sturio* (Williot *et al*., 2008). Under the scope of the project 'European action plan for sturgeons' developed by the World Sturgeon Conservation Society and WWF efforts and strategies for the development of captive reproduction protocols and habitat restoration have been successfully applied in the last decade.

Although most of endangered populations have low chances of survival without breeding and restocking plans, *ex-situ* conservation programs are challenging (Sousa-Santos, 2014). Changes in the original genetic pool due to inbreeding (i.e. favouring unrelated mating pairs) and the reduced ability shown by captive-bred fish to react to environmental stimuli, after releasing and therefore compromising post-release survival are the major concerns regarding this technique (Blanchet *et al*., 2008). Thus, these protocols should be developed without mutated natural selection pressures and by preserving as much as possible the original genetic diversity of the wild type population (Sousa-santos *et al*., 2014).

To mitigate the possible genetic depletion during captivity time, these programs should undertake the initial breeders as high as possible (Tenhumberg *et al*., 2004) and the captive period should be short with the least number of generations originated from the wild stock (Sousa-Santos *et al*., 2014).

The captivity time is influenced by the habitat restoration period. Thus, it is important that the elimination of threats in the wild habitat do not exceed the period of captivity corresponding to that which maintains the genetic integrity of the species. Nonetheless, longer period of captivity can lead to high fish densities on the tank and promote eggs and larvae cannibalism and higher susceptibility to disease and pathogen transmission (Sousa-Santos *et al*., 2014).

Due to artificial selection on captivity facilities, undesirable genetic and phenotypic consequences can negatively affect the *fitness* of fish and contribute for maladaptive behaviours upon their introduction in the wild habitat (Christie *et al*., 2012).

Environmental enrichment consists of increasing the physical complexity of the rearing environment and aims to simulate natural selection events during the captivity time. Either through the introduction of physical structures or changes in the feeding regime, the application of environmental enrichment has been shown to have positive effects on the development of fish natural behaviours (such as reproductive behaviour, seeking for shelter or predation interactions) (Näslund & Johnsson, 2016). In fact, Milot *et al*., (2013) compared the performance of Atlantic salmon reared in environmentally-enriched conditions (receiving natural prey and simulated predator attacks) with fish reared under standard conditions. The results suggest that environmentally enriched fish took longer to leave shelter, rather than take excessive risks (considered as a maladaptive behaviours of hatchery-reared fish).

The effects of environmental enrichment on captive rearing fish can change between species and other factors (Young, 2003) Nonetheless, there is a considerable variety of studies that support the positive effects of the environmentally-enriched conditions (Näslund & Johnsson, 2016), and therefore can be considered as a good strategy to attenuate or even eliminate the maladaptive behaviours originated during the captivity period. Research focusing the behaviour of the target species for *ex-situ* conservation protocols should be prioritized. Genetic monitoring analyses of the released fish and the wild type is crucial to understand the impact of this reproduction measurements (Fopp-Bayat, 2010).

In summary, to establish an *ex-situ* reproduction protocol, it is required to meet an equilibrium between some demands such as: (1) guarantee the maximum genetic diversity within rearing species, (2) not negatively affect the behaviour of the species and (3) proper space and human resources to monitor all the procedures (Gil *et al*., 2010). Since funding for freshwater conservation programs is often limited, zoos and aquariums play an important role to support these conservation actions by providing human resources and knowledge in captive breeding techniques (Reid *et al*., 2013).

3.1. Aquário Vasco da Gama project: *Ex-situ* reproduction of Portuguese endangered cyprinids in the context of their conservation

Aquário Vasco da Gama is a public institution dedicated to education, research, protection and conservation of freshwater and saltwater species. Was founded in 1898 by King Carlos I to commemorate the 400th anniversary of Vasco da Gama's arrival to India. Since 1919, AVG has been highlighted as an institution of high public value.

A pioneer program of *ex-situ* breeding of critically Portuguese endangered cyprinids was developed by AVG, ISPA-MARE (University), FMV (Faculty of Veterinary Medicine) and the Campelo Station (ONG Quercus facility) to ensure the survival of several populations of the endemic fish species to Portugal in imminent danger of extinction: *Achondrostoma occidentale, Iberochondrostoma almacai, Iberochondrostoma lusitanicum* and *Squalius pyrenaicus*

This project has been successfully carried out over the last 10 years and hundreds of individuals have been released in their natural habitat (*table I*).

Table I - **Captive breeding populations (number of individuals released) at the Aquário Vasco da Gama (AVG), between 2011 and 2019.**

Species	Population (River)	Number of individuals released
Achondrostoma occidentale	Alcabrichel	846
Achondrostoma occidentale	Safarujo	812
Iberochondrostoma almacai	Arade	976
Iberochondrostoma lusitanicum	Sado	1658
Iberochondrostoma lusitanicum	Lage	390
Squalius pyrenaicus	Colares	1070

To ensure the reproductive success and avoid the domestication of the species and all the constraints already described regarding captive breeding, this protocol follows a 'naturalistic approach' (Sousa-Santos, 2014) in order to not mutate the natural selection pressures. This method relies on: (1) outdoor tanks with natural light and conditions, fish were exposed to natural temperature oscillations and rainfall fluctuations, also crucial for gonadal maturation, (2) naturally occurring spawning: non hormonal induction or artificial fertilization are used, through environmental enrichment, fish were provided with identical spawning natural substrates such as gravel, plants and spawning mops, (3) availability of shelter and maternity areas for larvae and juveniles: no human intervention for protection of larvae, fry and/or juveniles, in order to promote the natural behaviour of finding shelter and avoiding predators and (4) minimal human intervention to minimize fish stress and prevent domestication.

Besides the reproductive strategies for this species, this project aims to guarantee the improvement of the wild habitat conditions. In fact, since the implementation of this project, a significant improvement of *A.occidentale* wild habitat has been accomplished.

Under the scope of the project 'POSEUR' supported by municipal authorities, some streams of Alcabrichel and Sizandro rivers have been rehabilitated, through bed and bank river cleaning and riparian cover rehabilitation techniques. Actions of environmental awareness with public entities such as schools have also been developed. Through the project 'Peixes Nativos', several activities have been established with students and teachers in order to contribute to the knowledge of the portuguese endemic fish and their main threats.

During this internship at AVG the target populations for this program were: *Achondrostoma occidentale* (Sizandro and Sarafujo River), *Iberochondrostoma lusitanium* (Sado River) and *Iberochondrostoma almacai* (Arade River).

3.2. Target Species and Habitat Description

3.2.1. *Achondrostoma occidentale* (Western Ruivaco)

Achondrostoma occidentale, also known as 'the western ruivaco' (Robalo, Almada, Sousa Santos, Moreira and Doadrio 2005) is a small cyprinid, whereby adults can reach a mean of 100 mm of total-length (TL). This species shares morphological and molecular features with two other *Achondrostoma* genus species (*A. arcasii* and *A. oligolepis*) (Robalo *et al*., 2005), being characterized by its greenish back coloration with an orange spot pigmentation in the anal, pelvic and pectoral fins.

A. occidentale has a reduced distribution range, in which only three independent populations are known to occur in lower stretches of three rivers located at western coast of Portugal: Alcabrichel, Sarafujo and Sizandro (*figure 2*).

These rivers have a temporary hydrological regime: this climatic pattern results in winter large floods and reduced or no flow in the summer. During the dry season (June - August), fish's survival is limited to a series of residual small disconnected pools under the shade of vegetation (Pires *et al*., 2014), whereby no individual can survive after extreme and completely drought. Indeed, population from Safarujo river was declared extinct until 2011 after an extreme drought (Sousa-Santos *no published data*). In the confined pools, the higher density of predators (such as *Procambarus clarkii and Micropterus salmoides*) is also threatening, specially to eggs and larvae (Lima *et al*., 2018).

Furthermore, these areas are heavily impacted by agricultural and domestic effluents, sewage from distilleries and pig farms. In fact, Teixeira *et al*., (2008) classified the water quality of the Alcabrichel River as contaminated (based on the results obtained for the biotic index on macroinvertebrates IBMWP). Nonetheless, a bad practice management of native riparian vegetation such as the arrow-leaved *Fraxinus angustifolia*, the white poplar *Populus alb*a and the willow *Salix salvifolia* is promoting habitat degradation. The constant removal of riparian vegetation either by governmental agencies (ARHTejo) and locals promote the change of thermal environment of these rivers. Also, riparian vegetation contributes as a substrate during the breeding season of this species (late April - May) since the breeders forms spawning aggregations and releases adhesive eggs over vegetation, small stones and gravel (Pereira, 2007; Robalo *et al*., 2005).

3.2.2. *Iberochondrostoma lusitanicum*

Iberochondrostoma lusitanicum (Collares-Pereira, 1980) is a small species (up to 148 mm TL) with elongated, flattened light brown body with some orange spots at the base of the dorsal fins. Shares some similar features with *I. almacai*, showing larger head with smaller eyes. The population of *I. lusitanium* has been reducing to about 80 % (Rogado *et al*., 2005), with a small fragmented distribution area: only occurs in the Sado River basin, in the lower part of the Tagus River basin and in the small streams with a moderate flow that flow into the sea north of Lisbon, up to the Lizandro River (*figure 2*). *Iberochondrostoma lusitanium* breeds in late April and May, forming spawning aggregations and releasing adhesive eggs over stones and vegetation. This species is subject to various pressures such as the implementation of hydraulic infrastructures, changes in flow regimes, extraction of riparian vegetation and the introduction of exotic species (Rogado *et al*., 2005).

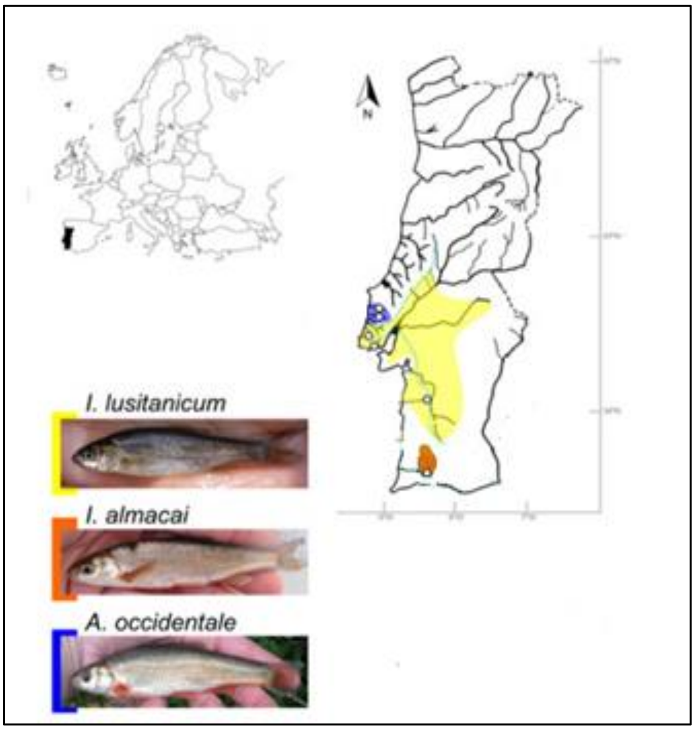

***Figure 2* - Wild distribution area of the target species for *ex-situ* conservation program at AVG.** (Adapted from Sousa-Santos *et al*., 2014)

3.2.3. Iberochondrostoma almacai

Iberochondrostoma almacai (Coelho, Mesquita & Collares-Pereira, 2005) reaches a maximum of 145 mm TL. This species is closely related to *Iberochondrostoma lusitanicum* being distinguished by his light brown body with paler underparts, dark pigmentation on the back and a smaller head with a downturned mouth and larger eyes.

Iberochondrostoma almacai shows a fragmented and restricted distribution: Mira, Arade and Bensafrim basins (*figure 2*). This species is found in deep areas with relatively warm water temperatures However, during the breeding season (January – April) the spawning usually occurs in shallow riffles located in fast-flowing water (Santos *et al*., 2008).

These rivers, especially the Arade basin, face severe anthropogenic pressures such as the existence of 3 hydroelectric power plants (Arade, Funcho and Odelouca), that have changed the hydrologic regime from lotic to lentic leading to a significant reduction of the habitat area. Also, these streams are highly disturbed by channelization followed by an introduction of exotic species such as the *Micropterus salmoides* (Santos *et al*., 2008)

4. Objectives

This internship took place at the Aquário Vasco da Gama, located in Cruz Quebrada – Dafundo, Oeiras, and lasted 8 months (September 2018 to May 2019).

The main goal of this internship at Aquário Vasco da Gama was to acquire captive fish maintenance technical skills, with special focus on the *ex-situ* conservation protocol of critically endangered cyprinids *Achondrostoma occidentale* (Sizandro and Sarafujo River), *Iberochondrostoma lusitanium* (Sado River) and *Iberochondrostoma almacai* (Arade River). To contribute to the knowledge about the biology of the highly endangered cyprinid *A. occidentale*, the first preliminary description of the embryonic and early larval stage was performed.

5. Procedures of *ex-situ* conservation protocol of endangered portuguese cyprinids

5.1. Establishment of the founding generation (F0)

To establish the founding generation (F0), fish were electrofished in their wild habitat according to the requirements of the ICNF (Institute for Conservation of Nature and Forestry): only catches of a maximum of 30 individuals are authorized and are subject to a prior authorization request. Thus, the spawners were transported to indoor quarantine tanks and remain for an acclimation period of at least 15 days. This period allows: (1) the reduction of stress caused during transportation (2) to determine the ability of individuals to adjust to the captive conditions, through the evaluation of their behavioural phenotype (Mayrand *et al*., 2019) and (3) the possible identification and removal of parasites.

Upon this time, sex and fork length (mm) is determined and fish are transferred to the breeding tanks. The F0 generation for all the populations in this captivity program was established before the beginning of this internship, thus the following tasks were carrying out on the populations described in *table II*.

Table II: **Captive breeding species populations at AVG:** Number of individuals and fish biometry values (mean fork length mm ± SD and the range min-max were determined during the *census* of 2018. *No data available for *A.occidentale* Sarafujo (SF).

Species	Population (River)	Number of individuals	Fish biometry (Fork length- mm)	Year of Foundation
A. occidentale	Safarujo (SF)	>338	-	2016 (F2)
	Sizandro (SZ)	>28	100 ± 1 (75-130) (n=28)	2017 (F1)
I. almacai	Arade (AR)	1379	83 ± 28 (37 - 128) (n=37)	2016 (F2)
I. lusitanium	Sado (SA)	286	73 ± 29 (30 - 120) (n=53)	2016 (F2)

5.2. Maintenance and reproduction conditions

5.2.1. Breeding tanks and environmental enrichment

Each brood stock was kept in 4 outdoor polyurethane and epoxy-resin concrete tanks with the following conditions: *A. occidentale* (SF) and *I. lusitanium* (SA) were kept in 3120 L tanks (500 x 195 x 32 cm); *A. occidentale* (SZ) and *I. almacai* (AR) in 1250 L tanks (500 x 100 x 25cm). The brood stocks were exposure to the natural photoperiod (day/night and summer/winter).

The tanks were integrated in an open circuit and water supplied by Lisbon city water supply system (EPAL) with regulated water flow (~ 60 L/h). An air compressor (Rebie Typ SKG 200 – 2,02, IP 44, 50Hz) provided aeration for all tanks (*figure 3*).

In order to avoid the release of some biological material (such as eggs, fry or small individuals) to the natural environment, some mechanical filters and retention areas were attached to the tanks (*figure 4*).

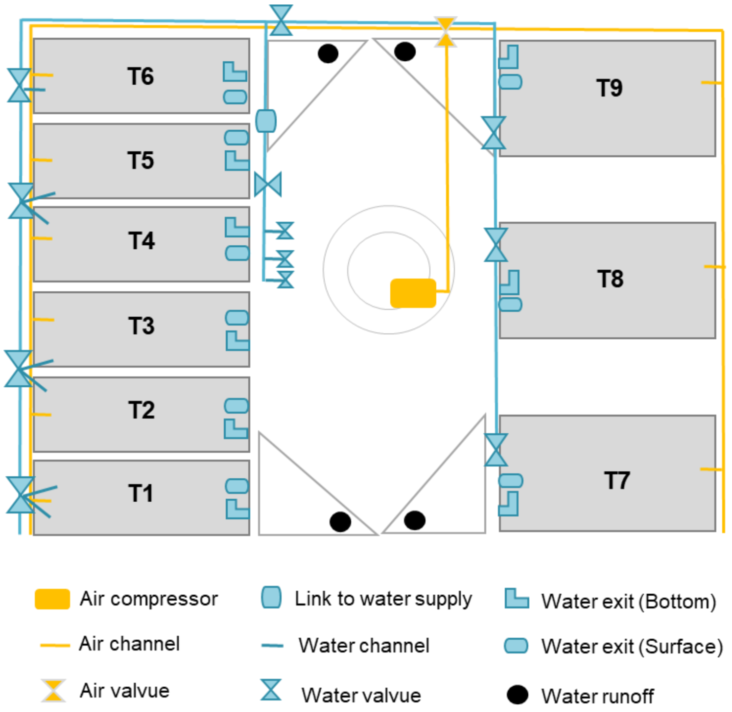

Figure 3 **- Scheme of the *ex-situ* conservation outdoor tanks at Aquário Vasco da Gama** (Adapted from an official AVG document).

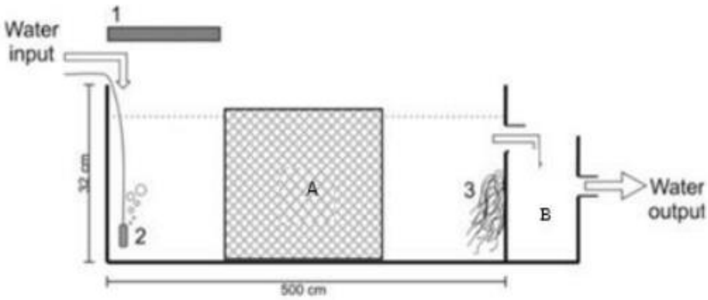

Figure 4 - **Experimental design of the breeding tank for *ex-situ conservation* project**: (**A)** Shelter area for juveniles; (**B**) Retention tank; **1**: Wood pier for shadow; **2**: Aeration; **3**: Spawning mop (Adapted from Gil *et al*., 2010).

Environmental enrichment (i.e. the presence of structures that promote the physical complexity to the rearing environment) is crucial to reduce the expression of undesirable traits that fish develop in captivity (Näslund & Johnsson, 2016).

Carvalho *et al*., (2002) demonstrated that *I. lusitanicum* spawning occurred when a male pressed the female against a physical substratum such as large stones or plant clutches. For *A. occidentale* aquatic vegetation is the preferred spawning substrate following the wool spawning mops (Mameri 2015). Following these preferences, the tanks were equipped with: (1) aquatic plant pots (*Cyperus sp*., *Echinodorus sp*., *Elodea sp*. and *Typha latifolia*) with abundant submerged roots, that also contribute as a shelter, a biological filter to maintain water quality and provides shadow to the tank; (2) wool mops placed near the aquatic roots to enhance the spawning behaviour and to facilitate egg observation; (3) a net cage (100 x 50 x 50 cm) and bricks in order to provide a shelter area for larvae and juveniles (*figure 5*). *I. lusitanicum* (SA) tank was also provided with gravel stones.

Figure 5 - **Environmental enrichment of the breeding tanks for *ex-situ conservation* project at AVG: (1)** Net cage; **(2)** Aquatic plant pots; **(3)** Wood pier and **(4)** Bricks. (Photographs by Cheila Almeida)

5.3. Tank Water Quality Management

Successful fish captivity depends on healthy fish and proper water quality management. Fish diseases and abnormalities usually occur after stress from poor water quality.

Reactive nitrogenous compounds (ammonia, nitrite and nitrate) are highly soluble in water. Ammonia is toxic to fish, especially in its un-ionized form (NH_3) and less toxic in its ionic form (NH_4^+). NH_3 can reduce fecundity, feeding activity and survival of fish as a result of negative physiological effects by causing asphyxiation, reducing the oxygen-carrying capacity, and affecting the immune system (Camargo & Álonso, 2006).

Nitrite (NO_2^-) is a byproduct of nitrification (oxidation of ammonia) and can cause severe fish physiological disturbances (Kroupova *et al*., 2005) . Nitrate (NO_3^+) is the final product and fish tolerate higher concentrations (Bregnballe, 2015) . The presence of abundant aquatic roots on the tanks contributes for the depletion of this compound, as the nitrate is usually the predominant form of available nitrogen that is absorbed by aquatic plants (Behrends, 2010).

Several factors affect the nitrification process such as water pH, temperature, concentration of dissolved oxygen, number of nitrifying bacteria present in the tank (Kroupova *et al*., 2005). Water pH is influenced by CO_2 concentrations (product of fish respiration and other organisms) and precipitation that naturally occur in the outdoors tanks .

Control and interpretation of the variability of these parameters are crucial to a good control overview of the tanks physicochemical dynamics and help to identify potentially problematic situation regarding fish growth and health.

5.3.1. Routines

For water quality management and control of the tanks the following parameters were monitored: temperature (ºC), water flow (L/s), dissolved oxygen (DO, mg/L), pH, redox potential (mV), hardness (ºF) and reactive nitrogenous compounds (Ammonia, Nitrite and Nitrate, mg/L).

Routine measurements for temperature, water flow, DO and pH were taken daily during the morning period. Temperature and DO were recorded trough a portable galvanic meter (HANNA HI9147) and pH measurements were taken with a pH-meter (E516 TITRISKOP METROHM). In addition, water temperature (ºC) and light intensity (lux = lumen per square meter) were recorded automatically every 4 h with a submerged datalogger (Hobo Pendant ® UA-002-08) placed in one of the tanks.

Reactive nitrogenous compounds (ammonia and nitrite), potential redox and hardness were determined once a week on the non-feeding days. For these parameters a sample from each tank was collected and analysed upon the application of the respective method (*Chapter 5.3.1.1. Methods*). After analysis, the value obtained for each parameter was checked to find it was within the reference limits. In addition, other procedures were performed such as checking the filters in the water outlets and restraint systems and the removal of excess visible feed waste.

5.3.1.1. Methods

i. Berthelot (Ammonia)

Ammonia analyses were performed by the Berthelot's method (Rhine *et al*., 1998). This method is based on the colorimetric quantification (at λ =640 nm) of indophenol blue dye in an alkaline solution from the reactions between NH4-N and Berthelot's reagents.

For this analyses 100mL of sample from each tank were prepared and mixture with 3 mL of Reagent 1 (Phenol and Sodium nitroprusside) and 3 mL of Reagent 2 (Sodium tricitrate and Hydroxide). The sample was stored in the dark for a minimum of 6 hours. Upon this time, ammonia concentration (mg/L) was determined by running the sample through a spectrophotometer WTW photoLab 6600 UV-VIS.

ii. Greiss (Nitrites)

Nitrites evaluation was performed trough the Griess method (Irandoust *et al*., 2013). Nitrite is detected upon the diazotization coupling reaction of nitrite by Griess reagents in hydrochloric acid solution at pH 1.0, that has a final product (purple dye) with maximum absorption at 545 nm. For this analyses, 50 mL of sample from each tank was mixed with 1 mL of Reagent 1 (Sulphanilamide Solution) and 2 mL of Reagent 2 (N-1-naphthyl) ethylenediamine dihydrochloride). The sample was stored for 10 minutes and upon this time nitrites concentration (mg/l) was determined by spectrophotometer (WTW photoLab 6600 UV-VIS).

iii. Brucine (Nitrates)

Nitrates analyses were performed by the Brucine method (Jenkins & Medsker,1964). This method is based on the colorimetric quantification at λ = 410 nm. For this analyses, 100 mL of sample from each tank was mixed with 2 mL of NaCl 30% and 0.5 mL of brucine acid reagent. This mixture was placed into a hot bath (above 95ºC) for 20 minutes. Upon this time the samples were replaced in a cold-water bath to lower the temperature to 15-20ºC. Nitrates concentration (mg/L) was determined by spectrophotometer (WTW photoLab 6600 UV-VIS).

iv. EDTA titration (Hardness)

Hardness analyses were performed by the EDTA (Ethylenediaminetetraacetic acid) titration. For this analyses, 50 mL of sample from each tank was mixed with 2 mL of buffer solution followed by the dye. Calcium (Ca^+) and Magnesium (Mg^+) ions formed a complex with the dye. During the titration with EDTA, Ca^+ and Mg^+ bind to EDTA, forming a more stable complex until a turning point (green colour solution). Hardness was determined through the conversion to French degrees (ºF).

5.4. Feeding

Growth and reproduction of fish are primarily dependent upon an appropriate supply of nutrients. Food must be provided on a regular basis for the steady growth of individuals contributing to the reduction of cannibalism. In addition, the type of feeding regime can also be a strategy of environmental enrichment.

The wild feeding behaviour of two Iberian species (Achondrostoma arcasii and Pseudochondrostoma duriense) was described by Sánchez-Hernández *et al.*, (2011).

The results suggest an omnivorous feeding habit for both species: feed mostly on detritus and plant material and aquatic macroinvertebrates (such as *Simuliidae*, *Chironomidae*). To provide a similar food source, fish were fed three times a week in the morning period, with a mixture of red mosquito larvae (*Chironomidae*) and *Artemia,* previously thawed (24 hours before administration to the tank).

To promote the search for prey, feeding was offered at different locations in the tank and the feeding behaviour was observed. This mixture sank into the tank and promoted fish forage throughout the water column rather than to the surface (considered as a maladaptive behaviour) (Tatara *et al*., 2008). The presence of aquatic plant pots provided vegetable nutrients sources (such as leaves, stems and roots). In addition, a mixture past (vegetable mix) containing shopped peas, carrots and mussels was developed. This mixture was offered frozen in order to avoid fragmentation in the tank, on the non-feeding days.

Feeding regime changed according to the following parameters: (1) fish density, (2) water temperature: in the winter fish metabolism slows and they tend to feed less, (3) food waste detected in the tank that contributes to the formation of nitrogen compounds and (4) water quality analyses.

This feeding regime was not applied to the early stages of life where no food was provided: fish larvae fed on phyto and zooplankton naturally occurring in the outdoor tanks.

5.5. Diseases control

When a dead individual (and/or with abnormal behaviour) was detected, a skin smear was performed and observed under a microscope for parasites identification (mostly, Platyhelminthes and protozoans were identified). For bacterial contamination detection, Faculty of Veterinary Medicine (Lisbon) conducts these analyses.

5.6. Tank transfers

As an annual routine, before the breeding season, all populations are transferred between the current tanks (origin tank) to a new tank (host tank). This allows tank cleaning and to determine the reproductive success of the current year for each species by performing the *Sensu* of each population (i.e. number of individuals) and population structure (number of adults versus juveniles, sex rate and adult maturation) and which individuals will be target for restocking.

The host tanks were previously cleaned, dried (during a period of 24 hours) and filled with the regular water flow. Water quality monitoring was performed (DO, Tº and pH) on both tanks. No feeding was performed for at least a 48 hours period before fish manipulation.

At the day of the transfers, the water level in the origin tanks was lowered to remove and replace all the environmental enrichment structures (i.e. bricks, plant pots, net cage) to the host tank. This procedure should be done carefully in order to identify if any spawning event has occurred and if there are eggs on the structures; upon this, the fish from each tank were caught with an extendable fishing net, transferred and maintained in aerated 50L containers (filled with water from the respective host tank for acclimatization). A sample of *n* individuals from each tank was measured (fork length, mm), weighted (total body weight, g) and sexed (*figure 6*). Females were identified by their abdominal distension, whereas males were recognized by fusiform body.

Figure 6 - **Procedures of tank transfers**: **A** - Origin tank cleaning; **B** - Aerated containers; **C** - Measurement of the fork length (FL, mm) (Photographs by Cheila Almeida).

5.7. Tag system

The fish used for restocking rivers were individually marked by the fin clipping method (partial abstraction of the dorsal fin). After fin regeneration, scars from the horizontal cut are still visible and were used to identify recaptures (*figure 7*).

This method has several advantages compared to other tag system such as: (1) easy and cheap to execute for a high number of individuals; (2) there is no risk of tag loss; (3) anaesthesia is not required; (4) fin clipping does not affect the survival or growth rate of the marked individuals (Thompson *et al*., 2005, Hand *et al*., 2010). Nonetheless, in order to avoid the stress caused by the handling it must be performed by experienced individuals.

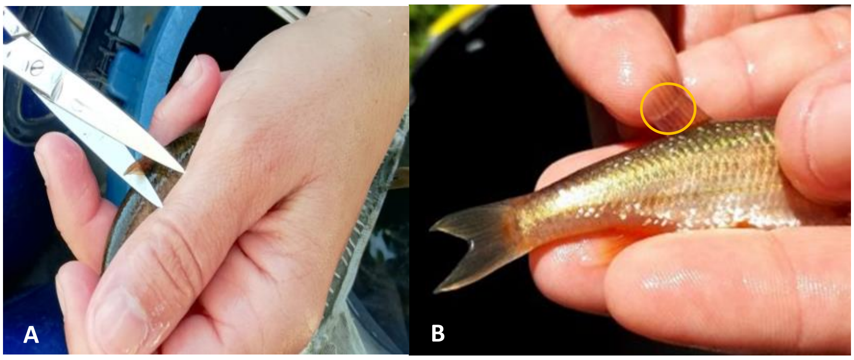

***Figure 7- Fi*n clipping method for the identification of the individuals released:**
A: Correct fish handling for partial fin abstraction; **B**: Scar after dorsal fin regeneration. (Photographs by Cheila Almeida (A) and Carla Santos (B))

5.8. Wild habitat pre-evaluation

The complete rehabilitation of natural habitats can be complex and can take indefinite periods, affecting time and location of restocking. A prior assessment of streams where restocking actions will be conducted is necessary, to verify if there are occasional sources of pollution, to check the stream river pool depth and length, the caudal velocity, instream coverage, riparian vegetation and detritus abundance (such as branches) to provide shelter areas and riparian vegetation (*figure 8*).

***Figure 8* - Wild streams pre-evaluation for fish restocking**: **(1)** instream vegetation coverage; **(2)** adequate depth and length and **(3)** detritus; From left to right: Ribeira de Odelouca and Safarujo River. (Photographs by Cheila Almeida)

5.9. Transportation and restocking

The captive populations target for release were determined after a prior analysis of the status of the captive and wild populations: If fish density on the captivity tanks exceed the ability to maintain species under ideal maintenance conditions and after a maximum of three consecutive generations in captivity, all the captive bred fish are released;

Before, the restocking actions of the stream rivers, an authorization for releasing is required from ICNF (Institute for Conservation of Nature and Forestry). On this document must be discriminate: (i) which populations will be released, (ii) the origin river basin, (iii) the stream where the restock will be performed (iv) date, (v) number of individuals and (vi) transportation conditions.

Successful transport of fish is closely related to the condition of the fish on the outset (Sampaio & Freire, 2016). The process that involved the manipulation of the fish prior to transportation (i.e. tank transfers and tag system) were performed in order to minimized integument damage and stress. From previous experiences at AVG, it was observed that the transport of smaller fish led to mortality rates of 20% during transportation. Therefore, individuals less than 400 mm in fork length long were spared from handling and transportation.

Fish were not fed for a period of 48 hours before transportation. This allows to reduce the amount of organic matter and the reactive nitrogenous compounds excreted by fish during the transportation (Sampaio & Freire, 2016). Fish were transferred in a 1000 L cylindric transport tank with constant aeration (*figure 9*). Before transportation, this tank was filled with water provided by EPAL and remained for 24 hours with aeration. This period allows to check if any cracks exist in the tank and monitoring the water quality before the fish transfer. The transport material was also consisted of: 50 L containers, extendable fishing nets, small buckets, a flexible PVC hose pipe and water sampling containers.

Upon arrival at the river stream and before the releasing procedure, water quality assessment was performed: DO, Tº and pH were determined on the transport tank and river stream. A sample of water was also collected and stored in the cold for posterior nitrogenous compounds evaluation (methods described on *chapter 5.3.1.1*.).

For fish releasing, the 50 L containers were filled with the transportation water and fish were replaced in the containers through an extendable fishing net. These containers were manually transported to the river stream and slowly placed close to the stream water surface to allow the acclimation. After a small period, fish started coming out of the container voluntarily.

During this internship the following populations were released: *I. lusitanicum* (Sado), *I. almacai* (Arade) and *A. occidentale* (Safarujo). *Annex 2* describes the location, number of individuals released, and wild habitat conditions.

***Figure 9* - Apparatus for the transportation and fish release during restocking rivers action: (A)** 1000 L transport container and 50 L containers; **(B)** water transfer procedure and **(C)** fish release. (Photographs by Cheila Almeida)

5.10. Public Awareness Activities

During this internship it was possible to participate in public awareness activities integrated on this *ex-situ* conservation program. These activities aimed to raise consciousness about the conservation of this species and their wild habitat pressures.

After a brief description of the biology of this species and their conservation *status*, students from regional primary schools and governmental entities were present and participated directly on the fish release. Also, restocking activities were covered by the national media channels.

In addition, Aquário Vasco da Gama was visited by the photographer Joel Startore from National Geographic. Over the past 25 years, Joel have been documenting critically endangered species around the world in the context of the *Photo Ark* project. During his visit at AVG, several species were photographed including the *Achondrostoma occidentale* and *Iberochondrostoma almacai* for further publication. Drafts of the final publication were provided by the photographer's team to include on this report (*Annex 1*).

6. Description of embryonic and early larval stages of *Achondrostoma occidentale*

Achondrostoma occidentale (Western Ruivaco) is an endemic cyprinid to Portugal listed as '*Endangere*d' by IUCN (Freyhof & Kottelat, 2008). This species has a reduced distribution range, in which only three independent populations are known to occur in lower stretches of three rivers located at the western coast of Portugal: Alcabrichel, Sarafujo and Sizandro. *A. occidentale* populations faces an increased risk of extinction due to the geographical isolation and severe habitat degradation (pollution, water abstraction and habitat destruction) (wild habitat description of *A. occidentale* on *chapter 3.2.1*.).

As an extreme protection measure, *A. occidentale* have been one of the target species of the *ex-situ* conservation program at Aquário Vasco da Gama. The main goal of this program is the restocking of the streams where this species naturally occurred.

The adoption of conservation efforts towards the protection and conservation of critically endangered species are generally supported by ethological data (Mameri, 2015). In addition, this breeding programs can also be a valuable tool to provide multidisciplinary data on this endangered species. Reproductive behaviour and substrate preferences of captive *A. occidentale* population were described by Mameri, 2015. Data relaying on the first life stages remain lacking as for most of freshwater fish species (Aral *et al*., 2011),

Studies on the early development of fish are important as they yield information on the mechanisms of the development, nutritional needs and environmental influences (i.e. temperature ranges, photoperiod and oxygen concentration) (Korwin-Kossakowski *et al*., 2008) . Disruptions and/or abnormalities in development stages of embryos and larvae are frequently considered as indicators of alterations in the environment (Aral *et al*., 2011). These data are also useful to identify development changes between wild and captive broodstocks (Park *et al.,* 2017).

Evaluation of embryonic and larval developmental should thus be performed under optimal conditions to avoid bias due to unfavourable incubation and larval rearing conditions such as water quality, temperature, or rearing systems (Korwin-Kossakowski, 2008).

The present study will provide the first descriptions on the early life stages of *A. occidentale* (Sizandro population) that will enable the identification of this endangered species through future larval studies. The method for the observation of eggs and larvae is also described since this study was undertake trough a naturalistic approach (i.e. under natural conditions of spawning, photoperiod and temperature).

6.1. Material and methods

6.1.1. Egg and larvae collection

A. occidentale eggs (Sizandro) were detected on March 26th upon the first spawning event (naturally occurred in the tank). Fish were kept under natural maintenance and reproduction conditions (*chapter 5.2.*). Total number of adults in the tank was 27 and the mean fork-length was 123 ± 12 mm (min 88 – max 150 mm). Egg mass were observed on the synthetic wool mops and plant pots (*figure 10A*). The mops were replaced in the tank net cage to avoid predation and to ensure that other batches were not performed on the same mop. The eggs were transferred to a cylindric plastic basin (composed by net walls to allow the normal water flow) placed inside the net cage (*figure 10B*). To describe the development of the eggs, these were collected from the cylindric basin through a Pasteur pipette (with tip removed) and microscopically analysed every day (microscope equipped with a digital camera and software Motic Cam 5+).

For this study, "day 0" corresponds to the first day of observation after egg detection (March 26th). Egg size was expressed as the mean diameter (mean ± SD , mm). Two eggs were observed each day, photographed and recorded. Unfertilized eggs and dead embryos were removed regularly.

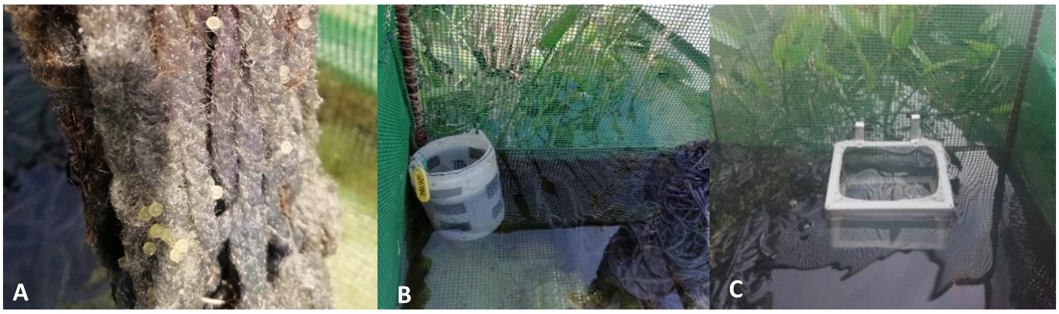

Figure 10* – Experimental *set-up* for egg and larvae collection*: *A. occidentale* eggs detected in the synthetic wool mops **(A)**; cylindric **(B)** and rectangular **(C)** basin (composed by net walls) for egg and larval collection, respectively. (Photographs by Cheila Almeida)

After hatching, the larvae were transferred to a rectangular basin composed by net walls (*figure 10C*). To observe the larval development, one larva was fixed and microscopically analysed daily until the 31st day. Upon this time, the observations were performed according to *figure 11*. Fish larvae and fry total length (mm) was measured from the tip of the snout to the edge of the caudal fin. Water temperature (ºC) was recorded by a portable galvanic meter (HANNA HI9147).

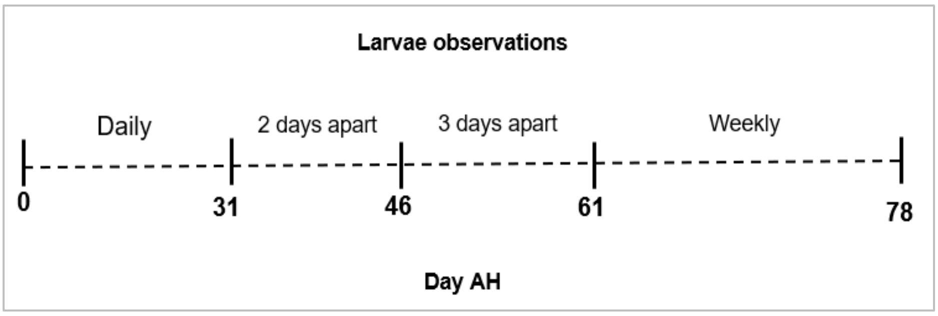

***Figure 11* – Scheme of time scale for *A.occidentale* larvae observations.**

6.2. Results

6.2.1. Embryonic development

From fertilization to hatching, sequence of the most important events of the embryonic development of *A. occidentale* (Sizandro) is shown in *Table III* and *Figure 12* .

a. Zygote and cleavage stage

The fertilized eggs (day 0) of *A. occidentale* were yellow, spherical in shape, adhesive and demersal. Diameters ranged from 1.7 to 2 mm and the mean diameter was 1.8 ± 0.1 mm. The egg membrane was thick composed by one layer with perivitelline space and visible micropyle. At day 0 of the embryonic development several cleavages had already occurred leading to the formation of the blastodisc.

b. Organ differentiation

Two days PF, notochord and somites were visible. Auditory vesicles vesicle, optic vesicle, tail vesicles were developed. The head and tail of the embryo were distinct. The first embryo movement was observed (small movements). Growth and development of the caudal area were evident. Caudal tail started to separate from the yolk (free trunk tail) and yolk mass was reduced.

Three days PF, the appearance of pigment could be observed in the eye lens. Vigorous movement of the embryo was observed as a result of repeated tail contractions. The heartbeat appeared between the third- and fourth-day post-fertilization. The rhythm of the heartbeat was about 103.5 beats per minute at the fourth day PF. The embryo was encircling the yolk sac.

Pigmented eyes and small spots on the inferior part of the tail were visible at this stage. A reduction in egg membrane thickness and small pigment spots were also observed. The flow of the red blood cells was visible.

Upon five days PF, the embryo occupied almost all the space in the egg. The embryo had vigorous movements. Nostrils structures and an apparent mouth were visible. A decrease in the thickness of the egg membrane was also observed.

Upon six days PF, embryo tail passed over the brain, showing slower movements. The heartbeat at this stage was 120 beats per minute. Between the seventh- and eighth-day PF, the number and size of the pigmentation spots on the surface of the tail and a yellowish coloration on the dorsal part of the embryo was observed. At this stage, the eyes were 0.4 mm (diameter) and had slower movements. The heartbeat was 133 beats per minute.

c. Pre-hatching

Eight days PF, the tip of the tail passed over the brain of the embryo and the tail was flattened. The egg hatched during the observation at 17,5º C. The acceleration of the body movements leads to a pressure against the egg shell. The larvae hatched tail-first and showed slow movement.

Table III - ***A. occidentale*** **embryonic stages and ontogenetic events description**: Day of observation post-fecundation (PF), temperature (T, ºC) and egg size (mean ± SD, mm).

Day PF	T (ºC)	Egg size (mean ± SD , mm)	Ontogenetic events	Stage
0	16.3	1.8 ± 0.1	Yellow and spherical egg; egg membrane composed by one layer with perivitelline space; meroblastic cleavage.	**Zygote and Cleavage**
2	15.5	1.8 ± 0.1	Cephalic differentiation and organization; notochord, optic vesicle and somites; separation of caudal section from the yolk (free trunk tail); yolk mass reduces; early movements.	**Organ differentiation**
3	15.5	2 ± 0.0	Developed eye lens with pigmentation; vigorous movement; Visible red blood cells flow.	
4	18.7	1.9 ± 0.1	Heartbeat (103.5 beats per minute), reduced yolk mass; pigmented eyes and tail; embryo was encircling the yolk sac.	
5	17.6	2 ± 0.2	Apparent mouth and nostrils; vigorous movement; heartbeat (114 beats per minute); decrease in egg membrane thickness.	
6	16.3	2 ± 0.1	Smaller yolk mass; increased heartbeat (120 beats per minute); slower movement. embryo tail passed over the brain.	
7	17.5	1.9 ± 0.1	Reduced yolk mass; flattened tail; eyes with slower movement; Eyes were 0.4 mm (diameter).	
8	17.3	1.9 ± 0.1	Increased heartbeat (133 beats per minute); dorsal embryo colour (yellow); yolk with visible chromatophores.	**Pre-hatching**

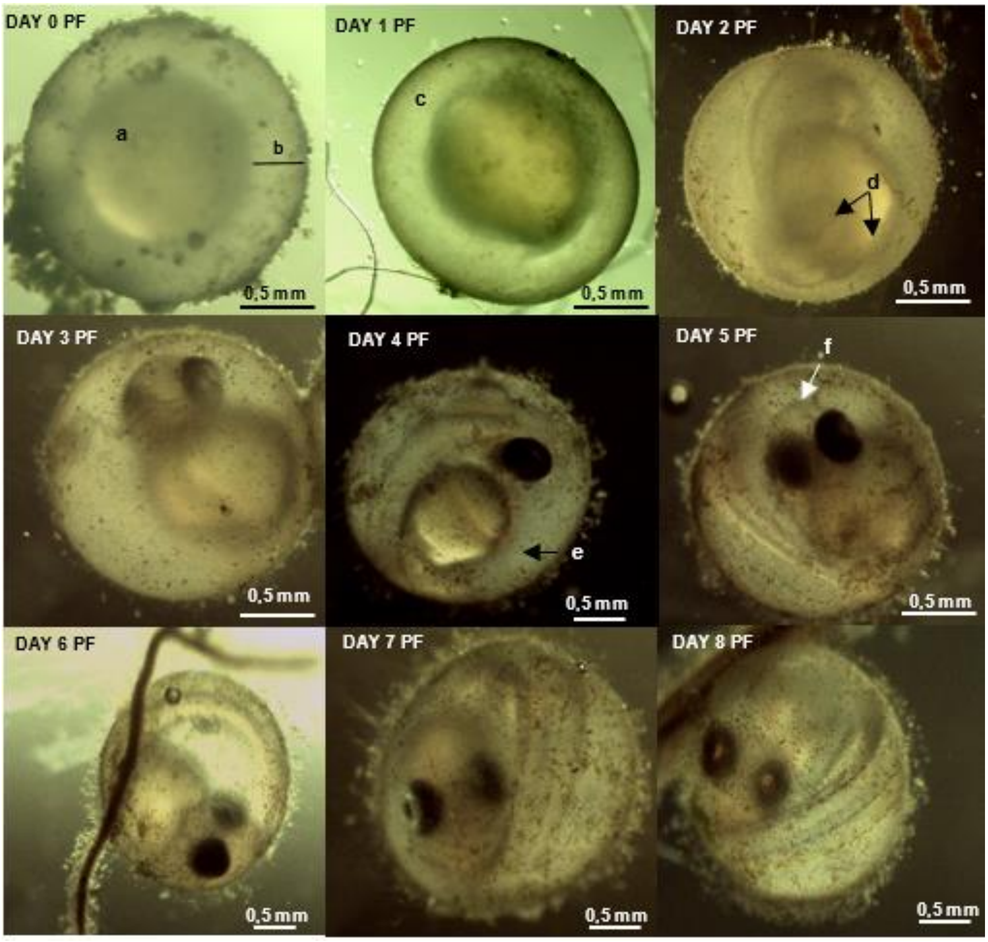

Figure 12 - **Embryonic development of *A. occidentale***. **Day 0** PF **(post fecundation)**: **(a)** micropyle; **(b)** perivitelline space; **Day 1 – 7** PF **(organ differentiation)**: **(c)** cephalic differentiation; **(d)** eyes lens; **(e)** red cells blood fluid; **(f)** nostrils; **Day 8** PF - (**Pre-hatching)** (Photographs were taken using Motic Cam software).

6.2.2. Larval development

The sequence of the most important events of the larval development of *A. occidentale* is shown in *Table IV* and *Figure 13 .*

One day after the hatching, *A. occidentale* larva was 7 mm in length. Head of the larva was bent toward the pyriform yolk sac. Mouth was developed but closed and intestinal tube was not fully extended, thus larva was still completely reliant on endogenous nutrients. Black chromatophores (melanophores) were visible on the larva's head, mostly located posterior to the eyes and extended to dorsal side. Three intern scattered melanophores were visible on the yolk sac and on the ventral side.

Three days AH, the total length of the larva was 7.4 mm. The total number of myomeres was 40, of which 15 were pre-anal. At this stage, the first swim bladder aerated completely, and larva improved the horizontal swim capability. The pectoral fin fold became easily observable. Pigmentation intensified, with lateral melanophores forming a line along the body, and the number of cephalic and ventral melanophores increased.

Upon six days AH, total length of the larva was 7.9 mm in length. At this stage, mouth was opened and algae on the digestive tract was visible. Therefore, larvae entered the transitional yolk-sac larval stage, relying both on endogenous and exogenous nutrients. The liver was formed.

Seven days AH, the yolk sac was completely absorbed. The larvae entered the stage of complete reliance on exogenous nutrients and therefore will be refer as fry henceforth. The first swim blander was elongated in a pear shape. At this stage the number of melanophores on the head increased and one melanophore appeared on the snout area. Development of pectoral fins with ray's outline was also distinguishable.

Twenty days AH, the fry was 80 mm in total length. Increased number of circular melanophores over the head and snout area, dorsal and ventral part. Two circular melanophores lie dorsally on each myomere. A distinguishable line over the inferior part of the auricular vessel and over the first swim bladder covered with circular melanophores and iridophores (iridescent chromatophores). Irregular melanophores were visible on the post-anal region. Irregular melanophores were present on the inferior part of the caudal fin over the ray's outline.

Forty-four days AH, the fry was 13.2 mm in length. At this stage, the swim-bladder was divided transversely into an anterior and a posterior chamber and fin fold reduced only to the pelvic area. Increased number of circular and irregular shaped melanophores and iridophores over the head and snout area, dorsal and ventral part.

Sixty-nine days AH, the fry was 21.5 mm in length. No fin folder was observed, and the swim bladder was long as the digestive system. Increased pigmentation (circular and irregular shaped melanophores and iridophores) over the head (smaller melanophores and snout area, dorsal and ventral part. Nostrils were divided in two parts and all the fins had completed and segmented rays.

Seventy-eight days AH, the fry was 29.5 mm in length. A completed urostilar torsion was observed and complete scales covered the body. At this stage, the fry was morphologically similar to a juvenile fish.

Table IV: ***A. occidentale* larval development**: Day of observation after-hatching (Day AH), temperature (T, ºC), total length (mean, mm).

Day AH	T (ºC)	Total Length (mm)	Development events
1	12.8	7	Larvae with pyriform yolk sac; mouth was developed but not open; melanophores over the head and dorsal area.
3	16.3	7.4	Swim bladder aerated completely, and larva improved the horizontal swim capability; myomeres visible (total of 40 - 15 were pre-anal and 15 post); increased pigmentation.
6	15.5	7.9	Larvae entered the transitional yolk-sac larval stage, relying both on endogenous and exogenous nutrients. The liver was formed.
7	18.9	7.9	Yolk sac was completely absorbed; first swim blander was elongated in a ‘pear’ shape; pectoral fins distinguishable with ray’s outline.
20	18.7	80	Increased number of circular melanophores and iridophores; caudal fin with distinguishable ray’s outline.
44	17.6	14	Swim bladder division; increased number of circular and irregular shaped melanophores and iridophores.
69	16.3	21.5	No fin folder; swim bladder was long as the digestive system; nostrils were divided in two parts; fins were segmented with complete rays: 8 pelvic rays, 16 pectoral rays, 11 anal, 10 dorsal and 38 caudal rays.
78	17.5	29.5	Completed urostilar torsion and complete scales covering the body. Fry morphologically similar to a juvenile fish.

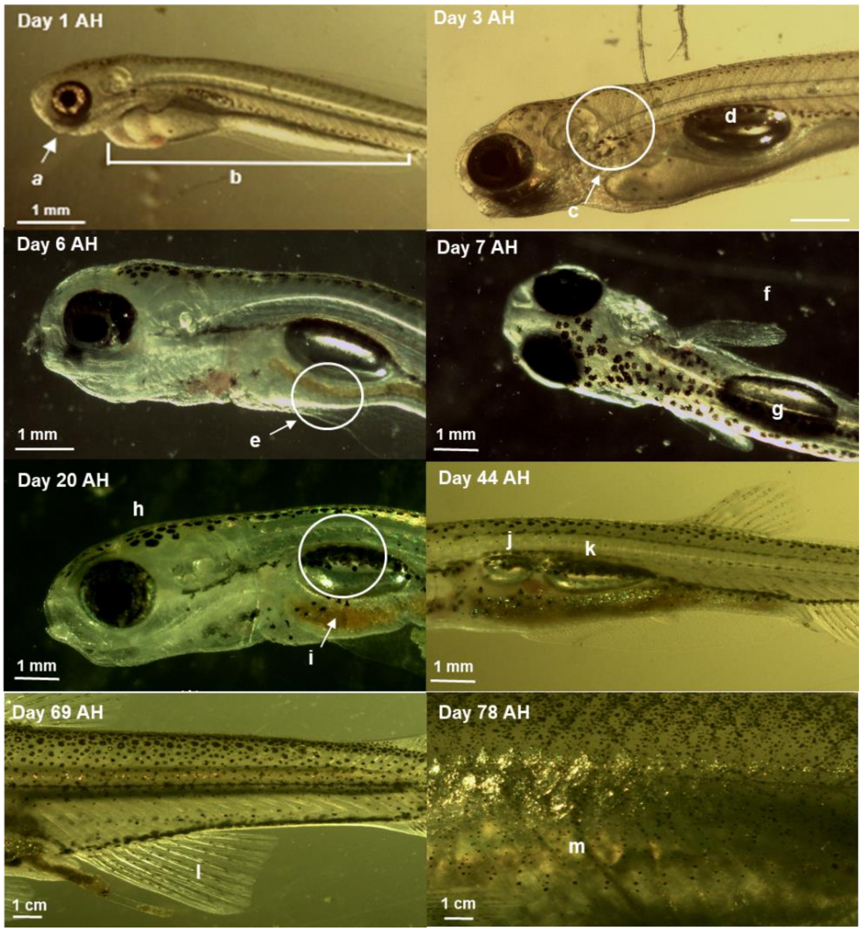

***Figure 13* - Important events and structures of *A. occidentale* larval development**. **Day 1** AH: **(a)** closed mouth, **(b)** pyriform yolk sac; **Day 3** AH: **(c)** lateral melanophores, **(d)** full swim bladder aerated; **Day 6** AH: **(e)** food on the digestive tract; **Day 7** AH: **(f)** pre-pectoral fins with ray's outline, **(g)** elongated "pear shape" swim bladder; **Day 20** AH: **(h)** circular melanophores over the head and **(i)** the superior area of the swim bladder; **Day 44** AH: **(j)** anterior and **(k)** posterior swim-bladder chamber; Day **69** AH: **(l)** anal fin segmented with complete rays; Day **78** AH: **(m)** completed scales (Photographs were taken using Motic Cam software).

6.3. Discussion

This study constitutes the first description of the early developmental stages of the highly endangered Iberian cyprinid *A. occidentale*. Through the description of some important developmental events of the embryonic and larval development, this work aimed to contribute for the knowledge of the biology of this species and identify key moments where these species may be most vulnerable to changes in the wild habitat.

Breeding season for *A. occidentale* were described to occur between late April to June (Pereira *et al*., 2007). During this study, the first spawning event took place on late March.

Eggs of *A. occidentale* were detected in the environmental enrichment structures present in the tank, particularly on the synthetic wool mops and close to plant roots. This observation follows the results obtained by Mameri (2015), that highlights the important of specific physical structures on both captive conditions and wild habitat for the reproductive success of this species.

Eggs of *A. occidentale* were spherical, adhesive and yellow with mean diameter of 1.8 ± 0.1 mm. *A. occidentale* eggs were similar in size to the Iberian fish species *I. lusitanicum* (1.9 mm) (Carvalho, 200) (cited in Carrapato & Ribeiro, 2012) and also to *Cyprinus carpio* (1.82 ± 0.06 mm) (Park et al., 2017) and relatively larger than the Iberian fish species *Anaecypris hispanica* (1.4 mm) (Carrapato & Ribeiro, 2012) and other freshwater fish species (Zhu *et al*., 2018).

Eggs were composed by one layer with perivitelline space. The principal function of the perivitelline space is the storage of oxygen. Larger perivitelline space is linked to contribute as a defense against environmental disruptions, such as fluctuation in oxygen concentration in the water (Korwin-Kossakowski, 2008).

The time of embryonic development was 8 days under a mean temperature of 16.7 ± 1.85 ºC. *I. lusitanicum* and *A. hispanica* (Carrapato & Ribeiro, 2012) have shorter incubation period, 6 days and 3 days at 20ºC – 21ºC, respectively. Increased water temperature is often associated with shorter incubation time for some cyprinid, including *C. carpio* and *C. carassius* (Zhu *et al*., 2018).

Incubation period is different within fish populations and can be related to adaptations to local environmental factors, such as water temperature, dissolved oxygen, pH and hereditary disparity (Kupren *et al*., 2010). In addition, other factors such as egg size can influence hatching time, with some species larger eggs requiring longer incubation periods (Sado & Kimura, 2002).

Longer incubation period, known as ‘delayed hatching’, can be associated as a strategy to improve the ability of the larvae to adapt to their environment immediately after hatching (Zhu *et al*., 2018).

One day post hatching, *A. occidentale* larva was 7 mm in total length. *A. hispanica* larva was relatively smaller (5.0 ± 0.1 mm TL). Larval development was slower than *A. hispanica* (Carrapato & Ribeiro, 2012) and *L. delineates* (Pinder & Gozlan, 2004). Korwin-Kossakowski (2008) demonstrated that, temperatures higher than 28 ºC are the optimal temperature range for larval development in European cyprinids.

The full aeration of the first swim bladder can be linked to the begin of exogenous feeding (Pinder & Gozlan, 2004), as the horizontal swim capability improve and therefore the larvae can search for exogenous nutrients. This was observed for *A.occidentale*. The first swim bladder completed aerated (3 days after hatching) and algae on the digestive track was observed upon 6 days after hatching.

The time for the total absorption of the yolk sac after hatching, signalizes the metamorphosis of larvae into fry (Zhu *et al*., 2018) and is linked to accelerated growth and decreasing chances of predation in the wild habitat (Korwin-Kossakowski, 2008). Total absorption of yolk sac was observed upon 7 days after hatching. This result is similar for *A. hispanica* (Carrapato & Ribeiro, 2012.) and longer than the time observed for *C. carpio* (Park *et al*., 2017).

During the larval development an increased number of melanophores and iridophores was observed both on *A. occidentale* larva and fry. These results follow the results obtained for other Iberian freshwater fish genera such as *Iberochondrostoma* and *Anaecypris* (Carrapato & Ribeiro, 2012). Pigmentation increase during development appears to be related with behavioural changes, when fish larvae or fry start to exploit new environments and require camouflage from predators (Pinder & Gozlan, 2004).

The time of transition to juvenile fish for *A. occidentale* was 78 days after hatching with a total length of 29.5 mm under a mean temperature of 16.7 ± 1.85 ºC.

In summary, this study highlights the following events and features related to *A. occidentale* ontogenetic and larval development: (1) *A. occidentale* eggs diameter was 1.8 ± 0.1 with perivitelline space that can be associated as an adaptation to an oxygen depleted environment, (2) embryonic period of 8 days under a mean temperature of 16.7 ± 1.85ºC, (3) 1 day AH larva was 7 mm in total length with visible pigmentation, (4) full aeration of the first swim bladder occurred upon 3 days AH, (5) total absorption of yolk sac was observed upon 7 days AH and (6) 78 days AH *A. occidentale* fry was morphologically similar to a juvenile fish with 29.5 mm in TL.

Information and data regarding fish development is scarce. Research on reproductive ecology of the highly endangered Iberian freshwater fish is limited. This work contributed to the first data regarding the early developmental stages of *A. occidentale*.

Low number of eggs released during the spawning event (naturally occurred in the tank) were observed, relatively to previous spawning events. Since larvae were fixed and euthanized for observation and regarding the threatened conservation *status* of *A. occidentale* a low number of eggs (n=2) and larvae (n=1) was described per observation in order to not compromise the reproductive success of *A. occidentale.* Therefore, more research in needed to complement this data.

In order to compare egg development between the major species cited on literature, further embryonic development observations for this species should be performed within shorter period. In addition, the method used was based on a “naturalistic method” (i.e. under natural conditions of spawning, photoperiod and temperature) and enfaces the importance of avoiding bias on the description of the first stages of the life cycle due to rearing conditions.

7. Final Remarks

The main goal of this internship at Aquário Vasco da Gama, was to follow and describe all the procedures of the ex-*situ* conservation protocol for the highly endangered Iberian cyprinid species (*Achondrostoma occidentale, Iberochondrostoma lusitanicum* and *Iberochondrostoma almacai*) and contribute to new data on the early stages of development of *A. occidentale*.

Ex-situ conservation techniques can be considered a good strategy to safeguard the endangered Iberian species survival. However, this conservation efforts per se do not guarantee the survival of the species in their wild environment and should be applied as the last survival hypothesis for endangered fish. Thus, the elimination of the main threats should be prioritized and accomplished before the complete *ex-situ* reproduction time, to provide the ideal *in-situ* conditions for the released fish and the survival of the next generations.

The raise of public awareness, such as the involvement of educational institutions and public and private sectors can give support for the implementation and improvement on conservation management for these species. They can be characterized as *flagship species* to raise support for local biodiversity conservation and to contribute for a good management policy for Iberian freshwater resources in order to accomplish one of the goals of the EU Water Framework Directive - Good ecological status and protection for each river basin.

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9. Annexs

Annex 1 - **Public awareness activities at Aquário Vasco da Gama –** Photographer Joel Startore under the scope of 'Photoark project (National Geoprahpic)'. **(A)** Experimental *Set up*; **(B)** *I.almacai;* **(C, D)** *A. occidentale.*

Annex 2 - **Restocking conditions:** Release site, date, Number of individuals (N) and wild habitat environmental conditions.

Species	**Release site (Stream)**	**Date**	**N. individuals released**	**T (ºC)**	**DO (mg/L)**	**pH**	**Ammonia (mg/L)**	**Nitrites (mg/L)**
***A. occidentale* (SF)**	Sarafujo	April	846	11.3	7.8	7.87	0.115	0.011
***I. almacai* (AR)**	Odelouca	March	976	20.3	13.9	9.31	0.125	0.005
***I. lusitanicum (*SA)**	Grândola	March	1658	15.7	7.8	7.52	0.039	-